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The *C. elegans* Developmental Fusogen EFF-1 Mediates Homotypic Fusion in Heterologous Cells and In Vivo

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Summary

During cell-cell fusion, two cells' plasma membranes merge, allowing the cytoplasms to mix and form a syncytium. Little is known about the mechanisms of cell fusion. Here, we asked whether *eff-1*, shown previously to be essential for fusion in *Caenorhabditis elegans*, acts directly in the fusion machinery. We show that expression of EFF-1 transmembrane protein drives fusion of heterologous cells into multinucleate syncytia. We obtained evidence that EFF-1-mediated fusion involves a hemifusion intermediate characterized by membrane mixing without cytoplasm mixing. Furthermore, syncytiogenesis requires EFF-1 in both fusing cells. To test whether this mechanism also applies in vivo, we conducted genetic mosaic analysis of *C. elegans* and found that homotypic epidermal fusion requires EFF-1 in both cells. Thus, although EFF-1-mediated fusion shares characteristics with viral and intracellular fusion, including an apparent hemifusion step, it differs from these reactions in the homotypic organization of the fusion machinery.

Introduction

Membranes merge during endocytosis, exocytosis, organelle biogenesis, cell division, fertilization, organ formation, cell death, and viral infections (Blumenthal et al., 2003; Jahn et al., 2003; Earp et al., 2005; Podbilewicz and Chernomordik, 2005; Kielian and Rey, 2006). Little is known about the mechanisms of cell fusion. Hypotheses for the mechanisms of cell fusion include the protein-mediated membrane fusion model, in which specific cell fusion proteins act at the point of contact between the plasma membranes of two cells analogously to viral and intracellular membrane fusion (Blumenthal et al., 2003; Jahn et al., 2003; Shemer and Podbilewicz, 2003; Stein et al., 2004; Chatterjee et al., 2005; Chen and Olson, 2005; Earp et al., 2005; Podbilewicz and Chernomordik, 2005; Kielian and Rey, 2006).

Potential membrane fusion proteins must meet several gold standards to be defined as fusogens: first, genetics and in vitro biochemical assays must demonstrate that the protein is necessary for membrane fusion events; second, cell biological approaches must show that the protein is expressed and active at the fusion site; third, expression of the protein in heterologous cells must be sufficient to induce cell-cell fusion. While there are many candidate fusogens, only intracellular SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), membrane glycoproteins from different enveloped viruses, and type I proteins from nonenveloped reoviruses have passed these three tests (Hu et al., 2003; Earp et al., 2005; Top et al., 2005). Proteins implicated in developmental cell fusion include: first, membrane proteins with multiple transmembrane domains implicated in yeast mating and fertilization; second, single-pass transmembrane proteins with immunoglobulin-like domains involved in muscle myoblast fusion, bone macrophage fusion, and fertilization; and, third, syncytin, a single-pass membrane glycoprotein involved in placenta trophoblast fusion (Stein et al., 2004; Chatterjee et al., 2005; Chen and Olson, 2005). With the exception of placental syncytin, no developmental cell fusion candidate protein has been shown to fuse cells in culture to date. Syncytin may be a specialized case in that it likely arose from a retroviral glycoprotein that appeared late in mammalian evolution and is found only in primates (Mi et al., 2000).

In *C. elegans* hermaphrodites, 300 out of a total of 959 somatic nuclei reside in syncytial cells that originate through programmed and stereotyped cell-cell fusions in living embryos and larvae (Podbilewicz and White, 1994; Mohler et al., 1998; Alper and Kenyon, 2001). The transmembrane protein EFF-1 was identified in *C. elegans* as a candidate fusogen by using genetic screens (Mohler et al., 2002; Shemer et al., 2004). EFF-1 is conserved within nematodes, and homologs have not been found in other phyla (Shemer and Podbilewicz, 2003). EFF-1 has been shown to be necessary for most cell fusions in *C. elegans* (Mohler et al., 2002; Shemer and Podbilewicz, 2002; Shemer et al., 2004; del Campo et al., 2005). Several mutations in *eff-1* block cell fusion throughout development, and ectopic, in vivo expression of *eff-1* in nematode cells that normally do not fuse results in cell fusion (Shemer et al., 2004; del Campo et al., 2005). EFF-1::GFP has been shown to concentrate at embryonic sites of cell fusion (del Campo et al., 2005). However, *eff-1* has not yet been shown to fuse cells in a heterologous tissue culture system and thus lacks a key element for consideration as a true fusogen.

To explore the biological functions of *C. elegans* EFF-1 proteins, we expressed different isoforms in transfected insect cells. EFF-1 transmembrane isoforms efficiently fuse cells in vitro. EFF-1 must be expressed in both fusing cells to merge them. EFF-1 forms complexes on the membrane, and the extracellular domain of EFF-1 stimulates cell-cell fusion through interactions

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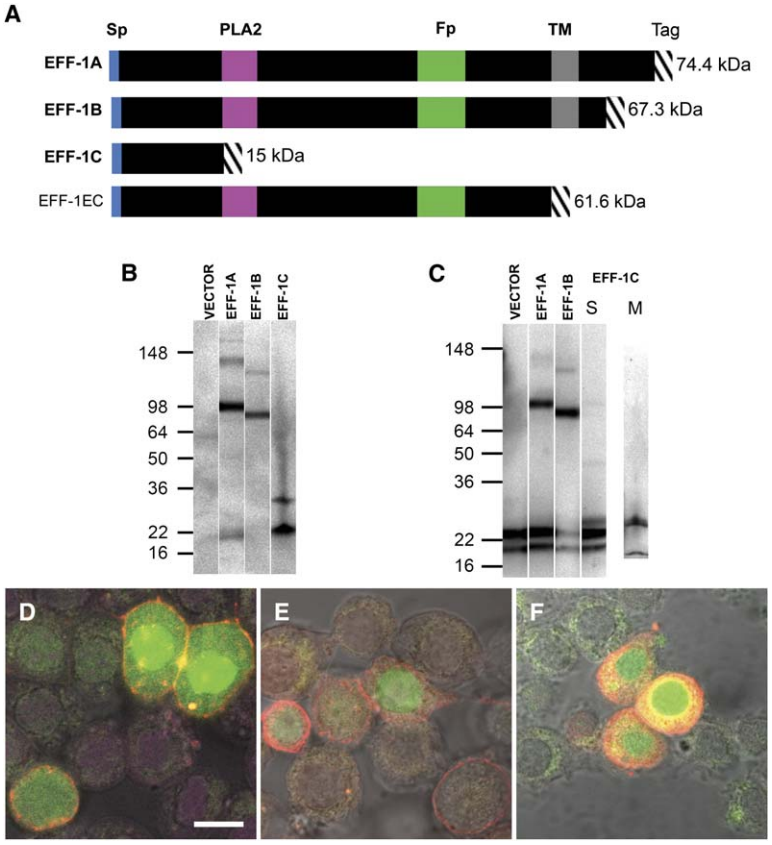

Figure 1. Expression of EFF-1-Tagged Proteins in Transfected Sf9 Insect Cells

(A) Isoforms EFF-1A, EFF-1B, and EFF-1C, all tagged with a V5-6×His epitope, were expressed in Sf9 cells. The isoforms are generated by alternative splicing. The extracellular and transmembrane (TM) domains of EFF-1A and EFF-1B are identical. The cytoplasmic tails of EFF-1A and EFF-1B are predicted to be 73 and 12 amino acids long, respectively, with no sequence similarity between them (Mohler et al., 2002; http://www.expasy.org/; TMAP, TMHMM, TMpred). The extracellular domain of EFF-1 was also expressed (EFF-1EC). Blue Sp, signal peptide that is removed from mature isoforms; pink PLA2, phospholipase A2 consensus active site; green Fp, putative fusion peptide; gray TM, transmembrane domain; hatched Tag, V5-6×His C-terminal tag.

(B) Western blot with anti-V5 antibodies on total lysates of Sf9 insect cells transfected with the empty vector (pIZT/V5-His), EFF-1A, EFF-1B, or EFF-1C.

(C) Surface biotinylation of transfected Sf9 cells followed by affinity purification with streptavidin agarose beads and Western blot with anti-V5 antibodies shows no specific immunoreactivity on VECTOR, a ~98 kDa band for EFF-1A, a ~85 kDa band for EFF-1B, and a minor band at ~25 kDa for EFF-1C. EFF-1C was detected in the medium (M), and some of it appears to stick to the cell surface (S).

(D–F) Immunofluorescence with anti-V5 antibodies (red) on (D) Sf9-EFF-1A, (E) Sf9-EFF-1B, and (F) Sf9-EFF-1C. (D) and (E) show surface expression of EFF-1A and EFF-1B, respectively. In contrast, the transfected cells in (F) show immunoreactivity primarily in intracellular compartments. Cytoplasmic GFP (green) identifies cells containing the DNA. The confocal images are representative of hundreds of fields in at least four independent experiments. The scale bar is 10 μm.

with membrane bound EFF-1 proteins. EFF-1 fuses cell membranes via hemifusion intermediates characterized by membrane mixing without cytoplasmic content merger. Since EFF-1 acts as a homotypic fusogen via hemifusion steps accompanied by the formation of complexes, these observations suggest a mechanism for the controlled formation of multinucleate cells in vivo.

Results

Expression of Three EFF-1 Isoforms in Sf9 Insect Cells

To study the activities of EFF-1 proteins in eukaryotic tissue culture cells, we expressed three EFF-1 isoforms in Sf9 insect cells derived from pupal ovarian tissue of *Spodoptera frugiperda* (Figure 1A). We chose these model cells for our studies because they normally do not form syncytia and they have been used for studies of cell fusion induced by viral fusogens (Chernomordik et al., 1995; Plonsky and Zimmerberg, 1996). We established transient expression of the alternatively spliced EFF-1A, EFF-1B, and EFF-1C isoforms tagged with V5-6×His epitopes and coexpressing cytoplasmic GFP (see Experimental Procedures and Figure 1). Western blot analyses on lysates of the different cells established that Sf9-EFF-1A cells expressed a major band of ~98 kDa. Sf9-EFF-1B cells expressed a single band with an apparent molecular mass of ~85 kDa, and Sf9-EFF-1C cells expressed a specific band with an apparent molecular mass of ~25 kDa (Figure 1B). The apparent molecular masses in Sf9 cells expressing EFF-1 isoforms were larger than predicted from the sequence (see Figure 1A), probably due to posttranslational modifications (e.g., glycosylation).

We determined the subcellular localization of EFF-1 proteins in Sf9 cells by using multiple approaches. First, surface biotinylation of transfected Sf9 cells, followed by streptavidin purification and immunoblotting with anti-V5 antibodies, showed that the EFF-1A and EFF-1B isoforms were expressed on the cell surface (Figure 1C). To determine whether EFF-1C was secreted, we loaded the media from cultured cells on denaturing gels. Western blotting with anti-V5 antibodies showed that EFF-1C, but not EFF-1A and EFF-1B, was found in the media, and that some of it may stick to the cell surface (Figure 1C and data not shown). Second, we detected expressed EFF-1 isoforms by using immunofluorescence (Figures 1D–1F). We found that EFF-1A and EFF-1B were localized at the plasma membrane and in internal organelles. In contrast, EFF-1C was found in intracellular compartments and not on the cell surface. Thus, the subcellular localization of EFF-1 proteins in transfected insect cells is consistent with their predicted structure, the predicted transmembrane proteins EFF-1A and EFF-1B are transported to the plasma membrane, and EFF-1C is partially secreted to the media.

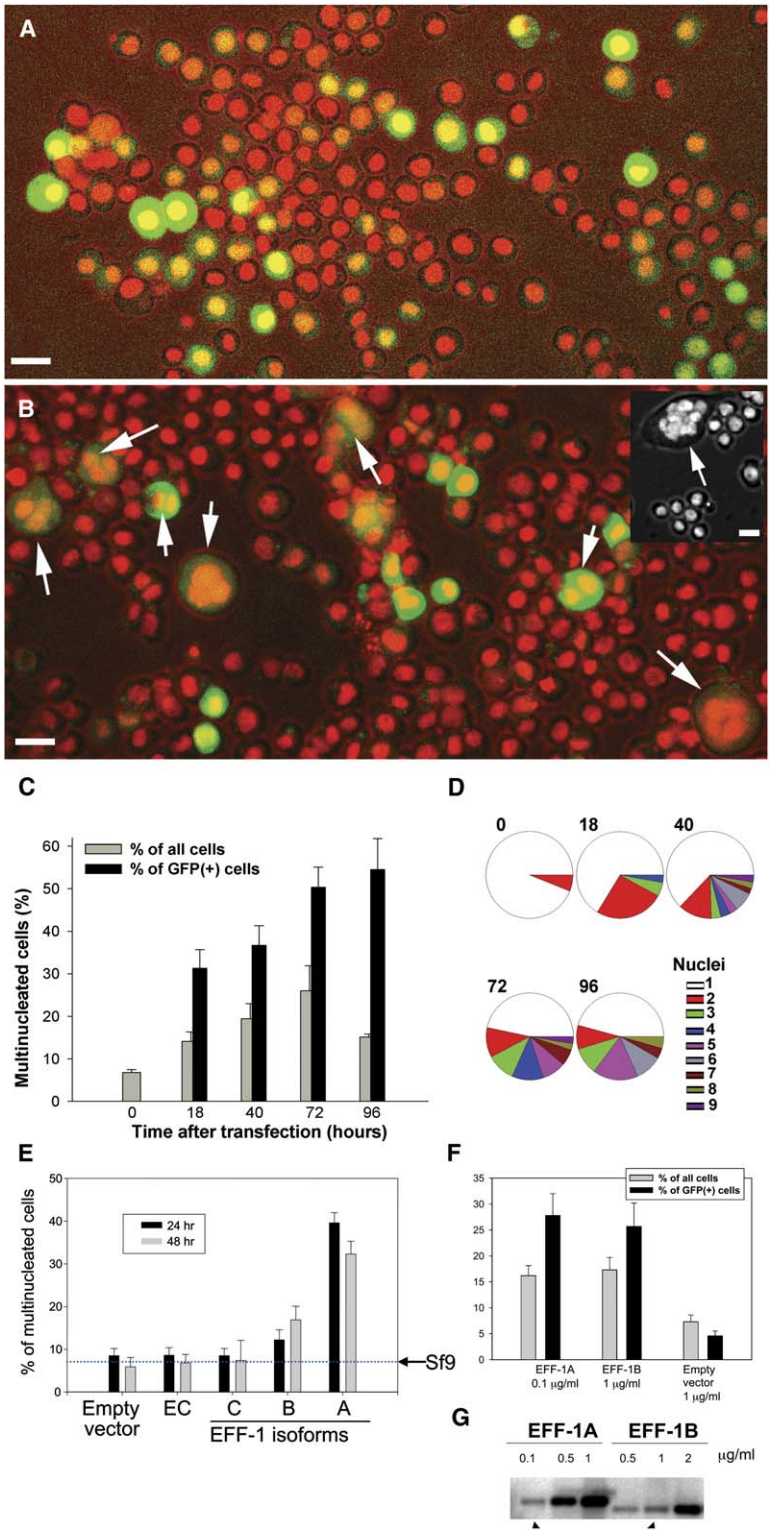

Figure 2. Ectopic Expression of EFF-1 Results in Multinucleation of Sf9 Cells

(A) Insect cells transfected with pIZT/V5-His empty vector show GFP expression (green) and nuclei (red; Hoechst).

(B) Transfected Sf9-EFF-1A cells become multinucleated (arrows). The insert shows a cell with ten nuclei (arrow).

(C) Kinetics of cell multinucleation in Sf9-EFF-1A-expressing cells after transient transfection shown as the ratio between the number of nuclei in multinucleate cells and the total number of nuclei (percentage of all cells; total nuclei: n = 1,437, t = 0 hr; n = 853, t = 18 hr; n = 790, t = 40 hr; n = 1,614, t = 72 hr; n = 940, t = 96 hr). The percentage of GFP(+) cells shows the ratio, taking into account only nuclei within cells expressing GFP (GFP(+) nuclei): n = 0, t = 0 hr; n = 235, t = 18 hr; n = 265, t = 40 hr; n = 223, t = 72 hr; n = 107, t = 96 hr.

(D) Distribution of the number of nuclei per GFP(+) cell over time (hr) for the same experiment shown in (C). Each pie shows the number of nuclei in cells with 1–9 nuclei divided by the total number of nuclei in green cells. For t = 0 hr, the percentage of all cells is given.

(E) Untransfected Sf9 cells show ~7% binucleate dividing cells. Sf9 cells transfected with empty pIZT/V5-His vector, the extracellular domain of EFF-1 (EC), or the EFF-1C isoform showed 7% ± 3% binucleate cells (normal cell proliferation). This is in contrast to cells that express EFF-1B (B) and EFF-1A (A), which showed an increase in mutlinucleation, with 2–11 nuclei/cell. All cells were transfected with 1 μg/ml DNA. We show the percent multinucleation of all cells (see Experimental Procedures).

(F) It required ten times more *eff-1B* than *eff-1A* DNA to obtain equivalent multinucleation. Percentage of GFP(+) cells (transfection efficiency): EFF-1A, 16.8%; EFF-1B, 24.3%; empty vector, 52.6%.

(G) Immunoblot after affinity purification of surface-biotinylated EFF-1 isoforms 48 hr posttransfection. Surface expression of EFF-1 isoforms is proportional to percent multinucleation.

Scale bars are 20 μm. Error bars indicate standard error.

Transmembrane EFF-1 Isoforms Induce Multinucleation in Heterologous Cells

To investigate whether any of the EFF-1 isoforms expressed in transfected insect cells were capable of inducing multinucleate cell formation, we looked for the presence of multinucleate cells at different times after transfection. We found that Sf9 cells that express EFF-1A or EFF-1B formed multinucleate cells starting 18 hr after transient transfections (Figure 2 and data not shown). Up to 60% of the cells expressing EFF-1A, detected as cytoplasmic GFP-positive cells, formed multinucleate cells containing 2–11 nuclei (Figures 2B–2D). In contrast, only 7% ± 3% of control Sf9 cells, Sf9-empty vector cells, Sf9 cells expressing the

extracellular domain (Sf9-EFF-1EC), and Sf9-EFF-1C cells formed binucleate cells (Figure 2E). These binucleate cells appeared to be dividing cells, since the nuclei were symmetrically positioned and had the same size and shape, and, often, the cells were observed undergoing cytokinesis (see Movie S2 in the Supplemental Data available with this article online). These findings show that while the partially secreted EFF-1C isoform did not induce multinucleation in insect cells, cell-surface expression of EFF-1A and EFF-1B yielded multinucleate cells (Figure 2E). It required ten times more *eff-1B* than *eff-1A* DNA to obtain equivalent surface expression resulting in comparable multinucleation (Figures 2F and 2G). Consequently, we used Sf9-EFF-1A for all subsequent studies.

C. elegans EFF-1 Fuses Heterologous Cells

To determine whether the formation of multinucleate cells by EFF-1A was a result of cell-cell fusion, we performed four tests. First, we obtained optical serial sections by using multiphoton laser scanning microscopy on putative multinucleate cells and confirmed that these cells were actually syncytial cells with a common cytoplasm (Figures 3A–3E; Movie S1). Second, we recorded time-lapse movies by using confocal microscopy and found Sf9-EFF-1-expressing cells that fused within 0.5 hr at 25°C (Figures 3F–3J; Movie S2). Within the same time frame, other EFF-1-expressing multinucleate cells divided and migrated, demonstrating that the multinucleate cells are physiologically active. Third, to determine whether multinucleate cells originate by EFF-1-dependent nuclear division without cell division (cytokinesis), we blocked the cell cycle and looked for multinucleate cells expressing EFF-1. We used 5-fluoro-2′-deoxyuridine (FdUrd) treatment to arrest Sf9 cells at the boundary of G1/S phase (see Supplemental Experimental Procedures). For the control Sf9-EFF-1A cells without FdUrd, we obtained multinucleation in 15.6% ± 2% of all cells (n = 811) and in 28.9% ± 2.4% of GFP(+) cells (n = 301). For Sf9-EFF-1A cells with FdUrd, we obtained multinucleation in 21.5% ± 3% of all cells (n = 792) and in 31.9% ± 2.9% of GFP(+) cells (n = 318). The finding that the transfected cells treated with FdUrd did not show a reduction in the number of multinucleate cells compared with untreated control cells is consistent with a mechanism of multinucleation independent from failure of cytokinesis. Fourth, we labeled the cytoplasm of some cells with Orange Cell Tracker and the cytoplasm of other cells with Blue CMAC Cell Tracker. After coincubation, we observed 139 EFF-1-expressing syncytial cells with both orange- and blue-labeled cytoplasm, demonstrating true cytoplasmic mixing (Figures 4A–4C, arrow; n = 825 cell contacts in 5 experiments). Taken together, these four tests demonstrate that EFF-1 expression in insect cells results in efficient cell fusion and syncytium formation.

EFF-1 Fuses Cells through a Hemifusion Intermediate

Protein-mediated fusion of biological membranes might start either with the opening of a proteinaceous pore, whose expansion leads to lipid merger (Han et al., 2004), or, alternatively, with the merger of only contacting leaflets of the fusing membranes into a hemifusion

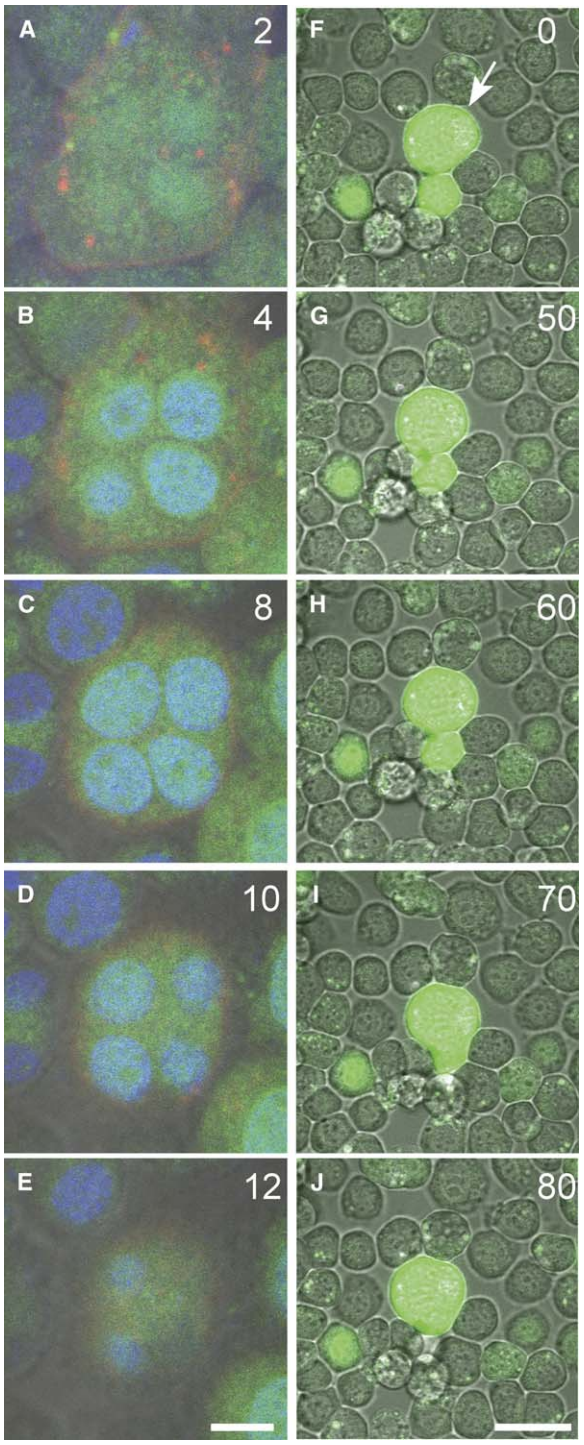

Figure 3. EFF-1 Expression Induces Syncytium Formation

(A–E) Immunofluorescence of a tetranucleate Sf9-EFF-1 cell analyzed by multiphoton optical sections. Shown are labeled nuclei (blue), co-expressing EFF-1 at the membrane (orange) and GFP in the cytoplasm (green). Numbers are optical sections in micrometers (see Movie S1). These images are representative of tens of multiphoton and confocal z stacks analyzed from multiple independent experiments.

(F–J) Time-lapse confocal frames showing cell fusion between a binucleate cell (arrow) and a mononucleate cell; numbers are in minutes from the start of the movie (see Movie S2). A higher-magnification z-series at the end of the recording confirmed that the cell had three nuclei.

Scale bars are 10 μm in (A)–(E) and 25 μm in (F)–(J).

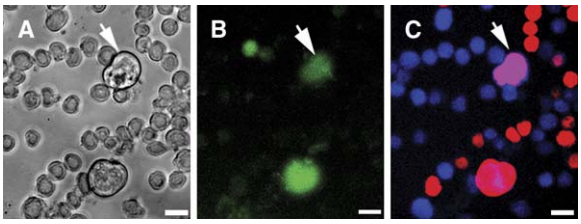

Figure 4. EFF-1 Expression in Sf9 Cells Mediates Cytoplasmic Content Mixing

(A–C) Half of the EFF-1-transfected insect cells were labeled with Orange Cell Tracker, and the other half were labeled with Blue CMAC Cell Tracker. After mixing, they were cocultured, and some giant multinucleate cells expressing GFP contained both colors, demonstrating that expression of transmembrane EFF-1 induces cytoplasmic content mixing and syncytium formation. (A) Phase view of giant cell (arrow). (B) GFP expression. (C) The giant cell contains both Blue CMAC and Orange Cell Trackers (arrow, pink cytoplasm). The images are representative of five independent experiments; n = 825 contacts between cells; 16.7% ± 2.2% had cytoplasmic content mixing (orange and blue).
Scale bars are 20 μm.

connection, which then breaks to form a pore (Chernomordik and Kozlov, 2005; Lu et al., 2005; Reese and Mayer, 2005; Xu et al., 2005). Early fusion intermediates are usually detected as outcomes alternative to complete fusion and are observed at suboptimal conditions. To test whether EFF-1 can promote hemifusion, we coplated transfected cells labeled with blue aqueous dye (Blue CMAC Cell Tracker) and transfected cells labeled with the red membrane dye DiI. In this fusion assay, we mixed the "blue" and "red" cells 18 hr after transfection. Two hours later, we looked for fusion events. At this relatively early time after coplating, only very few pairs of "blue" and "red" cells formed binucleate cells containing the red membranes and blue cytoplasm characteristic of complete fusion. However, 35 out of the 292 pairs of transfected cells (with cytoplasmic GFP) containing the red membranes and blue cytoplasm showed redistribution of the red lipid probe that was not accompanied by redistribution of the blue aqueous cytoplasmic probe (Figure 5A, cell pair marked by arrow and arrowhead). Thus, this experiment demonstrated the hallmark of the hemifusion phenotype in 12% of the cell pairs. In contrast, only 3 out of 739 pairs of untransfected wild-type cells showed the hemifusion characteristics. In summary, in addition to complete fusion, EFF-1 also promotes hemifusion.

Hemifusion and nonexpanding fusion pores in viral fusion can be transformed into full-grown fusion upon application of membrane tension generated by treatment of fusing cells with hypotonic medium (Chernomordik and Kozlov, 2003). To test whether we could stimulate the formation of complete fusion reactions in heterologous cells expressing EFF-1, we applied hypotonic medium to EFF-1-transfected cells and found a 20%–30% stimulation of complete fusion (Figure 5B). In contrast, control Sf9-empty vector cells ("No EFF-1") were not stimulated above the background number of dividing cells. Thus, hypotonic shock in EFF-1-expressing cells resulted in a significant ($p < 0.05$, t test) and specific increase in the number of syncytial cells. These experiments support the model in which early fusion intermediates, including hemifusion and reversible, nonexpanding fusion pores, transition into complete fusion.

An accepted method to assess whether hemifusion is a true intermediate in membrane fusion pathways is to reversibly inhibit this process by using a hemifusion-inhibiting lipid, lysophosphatidylcholine (LPC) (Chernomordik et al., 1993; Chernomordik and Kozlov, 2003;

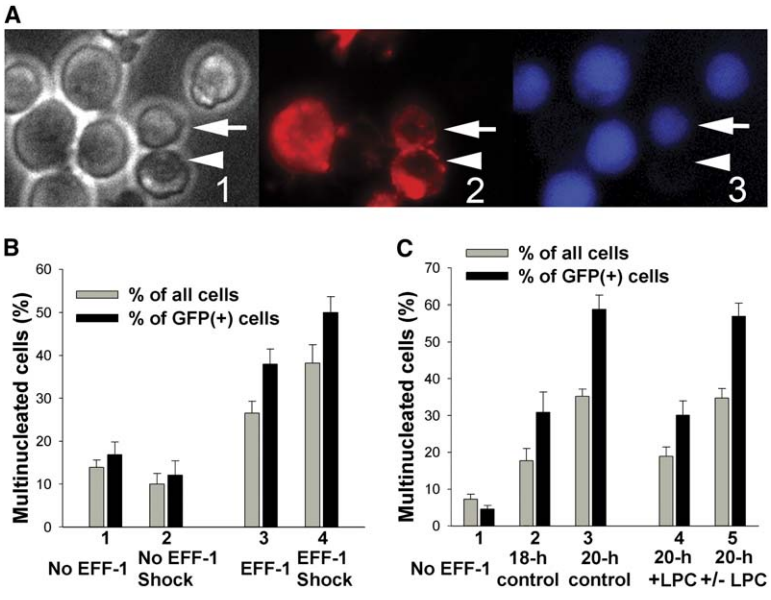

Figure 5. Evidence for EFF-1 Fusion through Hemifusion Intermediates

(A) Among EFF-1-expressing cells shown in phase contrast in (1), some cells, including the cell indicated with an arrowhead, were labeled with the red membrane dye DiI (2), and other cells, including the cell with the arrow, were labeled with cytoplasmic Blue CMAC Cell Tracker (3). Redistribution of the red membrane dye between the fusing cells in the absence of any transfer of the blue aqueous dye (red and blue fluorescence in images 2 and 3, respectively) defines the hemifusion phenotype. The punctate DiI red label inside cells is due to endocytosis.

(B) Membrane tension generated by application of hypotonic osmotic shock 18 hr posttransfection promotes fusion between EFF-1-transfected cells (bars 4 versus 3). This may occur by transformation of early fusion intermediates into complete fusion. In the control experiment (bars 1 and 2), osmotic shock did not increase the number of multinucleate cells among mock-transfected EFF-1(−) cells.

(C) An increase in the number of multinucleate cells observed between 18 and 20 hr posttransfection (bars 3 versus 2) is reversibly blocked by LPC applied 18 hr postinfection. Bars 4 and 5 represent multinucleate cells observed 20 hr posttransfection with LPC and after LPC removal, respectively. Bar 1 represents the control experiment with nontransfected cells. In (B) and (C), increases in the number of multinucleate cells upon cell fusion are shown as ratios between the number of nuclei in multinucleate cells and the total number of nuclei (percentage of all cells) and as similar ratios taking in account only nuclei within cells expressing GFP (percentage of GFP(+) cells).
Error bars indicate standard error.

Reese et al., 2005). To investigate whether EFF-1 mediates fusion through the hemifusion pathway, we tested fusion dependence on membrane lipid composition. In this experiment, we focused on an increase in the fusion extent observed for EFF-1-expressing cells between 18 and 20 hr after transfection. While application of LPC for 2 hr prevented this increase, subsequent LPC removal at 20 hr posttransfection resulted in fusion recovery. As it did with viral fusion and SNARE-dependent intracellular fusion (Chernomordik et al., 1993; Reese et al., 2005), LPC reversibly inhibited EFF-1-mediated cell fusion (Figure 5C).

Taken together, our results suggest that membrane fusion mediated by the developmental fusogen EFF-1 shares with viral and intracellular fusion reactions a common lipidic intermediate that may underlie all membrane fusion reactions. However, as reported in all published papers on different biological membrane fusion reactions, we cannot exclude the possibility that hemifusion is a dead-end branch of the fusion pathway rather than an intermediate in the productive fusion pathway. In spite of many excellent studies of viral and intracellular fusion, hemifusion is still a conjecture (Chernomordik and Kozlov, 2005; Lu et al., 2005; Reese and Mayer, 2005; Xu et al., 2005).

EFF-1 Interactions

EFF-1 transmembrane proteins may act alone or in complexes to fuse cells. To determine whether EFF-1 protein complexes are required to fuse cells, we first asked whether EFF-1 exists exclusively in the monomeric form. We ran lysates of Sf9 cells expressing EFF-1A tagged with V5-6×His on a 3%–8% gradient denaturing NuPAGE gel, and we detected a ladder of EFF-1A proteins with the anti-V5 antibody on Western blots (Figure 6A). Oligomers containing EFF-1A were stable under reducing conditions, but they can be dissociated by higher concentrations of denaturing detergents (2% SDS) or by prolonged boiling in sample buffer. Crosslinking of EFF-1A before lysis of the cells maintained the ladder and increased the intensity of the bands running at higher apparent molecular weights. These ladders are consistent with the existence of EFF-1A protein-protein interactions and the formation of complexes on the surface of Sf9 cells. To determine whether EFF-1A must be expressed on the surface of cells to form complexes, we expressed the extracellular domain of EFF-1 (EFF-1EC) in Sf9 cells. When we ran the conditioned medium from Sf9-EFF-1EC cells on the same denaturing gels with or without crosslinking, we found that EFF-1EC is able to form complexes in solution (Figure 6B). In contrast, EFF-1C did not form ladders (data not shown), suggesting that the C terminus of the extracellular domain of EFF-1 is required in order to form protein-protein complexes. We then asked whether EFF-1EC can form complexes after affinity purification. EFF-1EC expressed in transfected Sf9 cells contains the V5-6×His tag on its C terminus. It was secreted to the medium and was purified by use of Ni-NTA agarose beads. We found that monomeric EFF-1EC was purified on Ni-NTA beads, and that, following crosslinking with DMP, complexes appeared to migrate more slowly, suggesting the formation of dimers and trimers (Figure 6C). Thus, EFF-1 forms complexes in Sf9 cells and on beads,

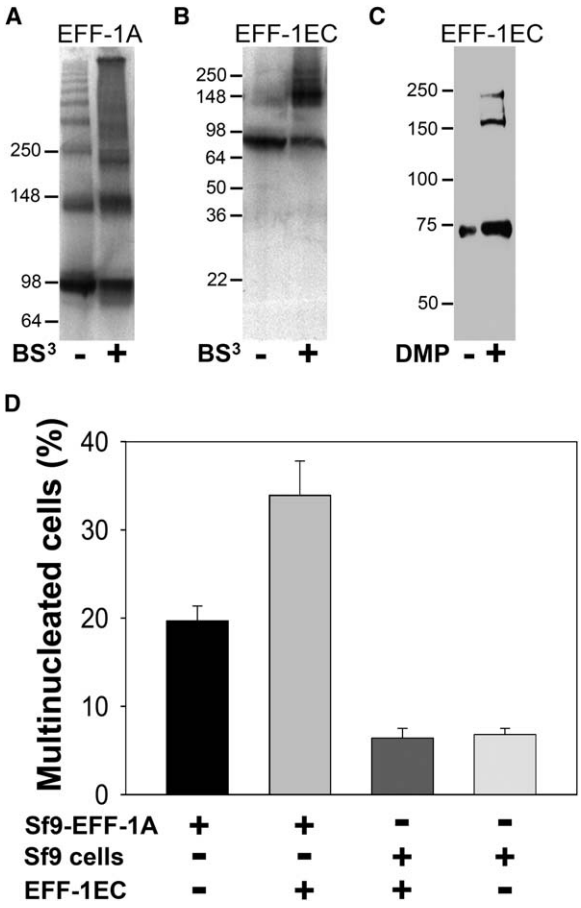

Figure 6. EFF-1 Proteins Interact, Forming Complexes during Syncytiogenesis

(A) EFF-1A proteins form complexes in Sf9-EFF-1A cells (ladder: ~74, 85, 147, 232, 287, 327, 356, 394, 399 kDa). The apparent high-molecular weight bands were obtained both with and without crosslinker. Crosslinking with BS^3 (Pierce) on intact cells results in a shift to lower-mobility complexes. We separated these complexes with denaturing 3%–8% NuPAGE gels and detected them by using immunoblot with anti-V5 antibodies as shown in Figure 1B.

(B) The extracellular domain of EFF-1 (EFF-1EC) is secreted to the medium by Sf9-EFF-1EC cells and forms complexes. After crosslinking with BS^3, most EFF-1EC shifts to bands of apparently higher molecular weights.

(C) EFF-1EC from the medium was affinity purified by using Ni-NTA agarose beads (~77 kDa). Crosslinking with DMP (Sigma) on the beads results in the formation of apparent dimers (~172 kDa) and trimers (~204 kDa). These complexes were separated with denaturing 8% SDS-PAGE gels and were detected by immunoblot.

(D) EFF-1EC stimulates EFF-1A-mediated syncytium formation. Control Sf9 cells with or without EFF-1EC show ~5% binucleate dividing cells. Error bars indicate standard error.

demonstrating that EFF-1 proteins can interact. To determine whether the recruitment of EFF-1EC to the surface of Sf9 cells expressing EFF-1A can affect the fusogenic activity of EFF-1A, we measured the formation of multinucleate cells and found that EFF-1EC stimulates EFF-1A-mediated multinucleation by 20%–35% (Figure 6D). These findings suggest that EFF-1 complexes on the surface of Sf9 cells efficiently mediate cell fusion. While the specific mechanisms by which interactions between EFF-1A and EFF-1EC promote fusion remain to be understood, we hypothesize that both proteins

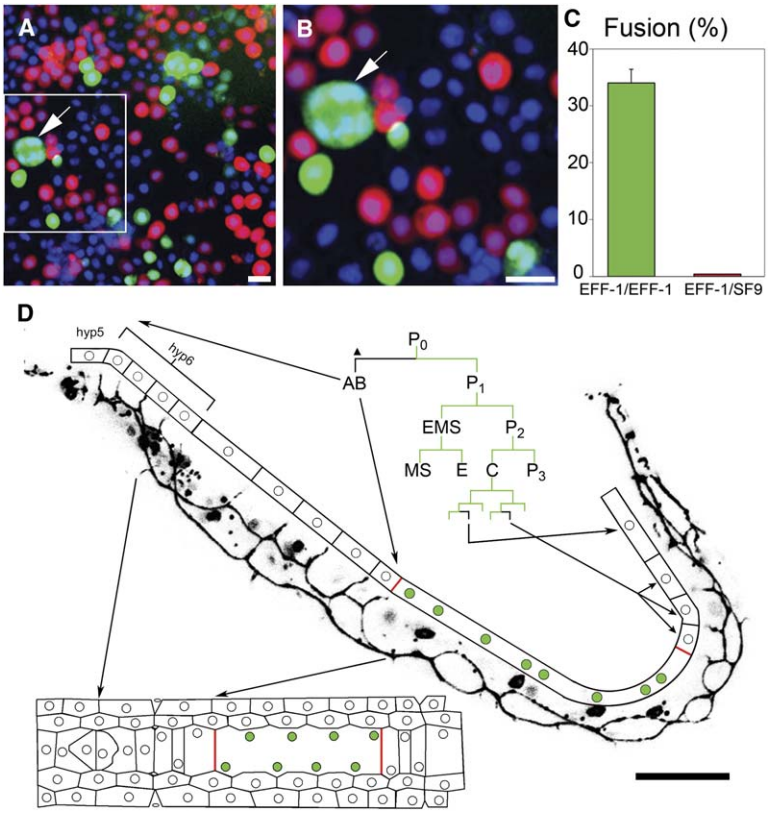

Figure 7. EFF-1-Mediated Cell Fusion Is Homotypic in Insect Cells and in *C. elegans* Embryos

(A and B) Cell-cell fusion is a homotypic process. Orange, EFF-1(−) Sf9 cells; green, GFP(+) EFF-1(+); blue, (A) nuclei. (B) Higher magnification of a region in (A) shows a multinucleate cell that is only green (arrow); orange cells do not fuse. Only green cells became syncytial.

(C) Quantitation of homotypic fusion 40 hr after transfection (green; n = 922). Only one fusion event between an orange EFF-1(−) cell and a green EFF-1(+)-expressing cell was identified (n = 506). Error bars indicate standard error.

(D) Mosaic analysis shows that *eff-1(+)* expression is necessary in both cells that fuse in *C. elegans* embryos. Lineal origins of epidermal (hypodermal) precursors of hyp5, hyp6, and hyp7 syncytia. Only early embryonic divisions are shown. In the embryo, hyp7 is formed by the fusion of 23 cells, 11 derived from AB and 12 derived from C. In the mosaic L1 larva shown, the array was lost three times: first in AB (black triangle) and two times in descendants of C. The result is a mosaic animal with a hypodermis that contained only 8 *eff-1(+)* and GFP(+) green nuclei (two are out of focus). Thus, only eight cells fused to form a syncytium, as is apparent in the L1 larva imaged in the confocal microscope showing a partial left dorsal view (negative image). Other nuclei of muscle and intestinal cells can also be seen in this partial projection. The drawing above is a dorsolateral view of the dorsal hypodermis. Red lines show the junctions between the small syncytium and the *eff-1(−)* cells. The drawing in the bottom is a cylindrical projection of the skin of the mosaic worm cut open in the ventral midline, showing the major epidermal cells and the small syncytium. This and other genetic mosaics show that both cell partners must be *eff-1(+)* to fuse (see Supplemental Data and Figure S2). Anterior is to the left.

Scale bars are 20 μm.

interact by developing a dense, interconnected protein coat around the fusion site (Chernomordik and Kozlov, 2003). It has been proposed that this fusion protein coat deforms the underlying lipid bilayers and produces tension that drives the expansion of the fusion pore.

Homotypic Organization of EFF-1-Mediated Cell Fusion

We then explored whether EFF-1 has to be expressed in both of the fusing cells or only in one of them. We mixed Sf9-EFF-1(+) cells (GFP(+) cytoplasm) with "innocent" EFF-1(−) untransfected Sf9 cells (orange cytoplasm) and found 1 fusion event out of 506 contacts between EFF-1(+) and EFF-1(−) cells. In contrast, for the same size fields of view, we found one third of all EFF-1(+) nuclei in syncytia (Figures 7A–7C). Thus, fusion between two EFF-1(+) cells is much more efficient than fusion between EFF-1(+) and EFF-1(−) cells.

Our finding that cells transfected with *eff-1* do not fuse with *eff-1(−)* cells was supported by a further analysis. As in the experiment shown in Figures 7A–7C, unlabeled EFF-1-transfected cells were coplated with innocent cells labeled with Orange Cell Tracker. We focused on the innocent cells in contact with either innocent or EFF-1-transfected cells. To obtain an upper estimate of the efficiency of fusion between transfected and untransfected cells, we assumed that all labeled binucleated cells observed in this experiment were the results of cell fusion. (In reality, most, if not all, of these binucleate cells observed in this experiment likely represented dividing rather than fusing cells). The efficiency of fusion between transfected and innocent cells was expressed as a probability of fusion per cell contact, $P_{EFF\text{-}1}$ ($P_{EFF\text{-}1}$ = 0.015, n = 1202 contacts). We compared $P_{EFF\text{-}1}$ with the probability of fusion between transfected (=unlabeled) cells per cell contact, $P_{EFF\text{-}1\text{-}EFF\text{-}1}$, observed in the same experiment ($P_{EFF1\text{-}EFF\text{-}1}$ = 0.075, n = 1532 contacts). If we assume as our null hypothesis that EFF-1 present in either one or two bound membranes fuses membranes independently with the same efficiency, EFF-1 expressed in only one of the two bound cells is expected to fuse cells with the probability $P_{EFF\text{-}1} = P_{EFF\text{-}1\text{-}EFF\text{-}1}/2$. Based on the binomial distribution analysis, the probability that even the upper estimate of $P_{EFF\text{-}1}$ given by our experiments can be explained within our null hypothesis is only 3×10^{-6}. Thus, our findings strongly indicate that EFF-1 is required in both fusion partners to induce efficient fusion in a heterologous tissue culture cell line.

These results provide experimental evidence for the models for homotypic EFF-1-mediated fusion in *C. elegans* that were based on the observation that EFF-1::GFP accumulates between cell pairs that eventually fuse (del Campo et al., 2005; Kontani and Rothman, 2005). This is in contrast to viral fusogens that merge membranes when expressed in one membrane, and

it is similar to the intracellular machinery that fuses membranes when vSNARE and tSNARE complexes are expressed in different membranes (Hu et al., 2003; Jahn et al., 2003; Earp et al., 2005).

Both Epidermal Cells Must Express EFF-1 to Fuse in *C. elegans*

To determine whether the homotypic model is also valid in *C. elegans* embryos, we did mosaic analysis of *eff-1*. Genetic mosaics were generated by spontaneous loss of an extrachromosomal array during embryonic cell divisions (Herman and Hedgecock, 1990; Yochem et al., 1998; Myers and Greenwald, 2005). In these animals, both chromosomes II carried a null allele of *eff-1*; an extrachromosomal array carrying *eff-1(+)* and an independent cell marker (nuclear *sur-5::GFP*; Yochem et al., 1998) allowed us to determine when and where the array had been lost in the early embryonic lineage (Figure 7D; Figure S2). In addition, the strain had an integrated transgene that specifically expresses the apical junction marker AJM-1::GFP and conveniently identifies unfused epithelial cells that normally fuse in the hypodermis (Shemer and Podbilewicz, 2002). Animals that completely lost the *eff-1(+)* array maternally had 100% epidermal cells that failed to fuse (n > 500). In contrast, animals had a completely rescued and normal cell fusion phenotype when all epidermal cells carried the *eff-1(+)* array (n > 200). We found mosaics that lost the *eff-1(+)* DNA in some precursors of epidermal cells. In these mosaic animals, cell fusion was detected only between pairs of cells, both of which contained GFP-positive nuclei (eff-1(+)/eff-1(+), n = 60). In the same mosaic worms, we found 55 eff-1(−)/eff-1(−) pairs that failed to fuse and only 3 eff-1(+)/eff-1(+) pairs of cells that failed to fuse, probably because of a low EFF-1 dosage in one of the partners, which appeared weakly GFP positive. When eff-1(+) cells were in close contact with eff-1(−) cells, cell fusion did not occur (n = 15; Figure S2). Thus, *eff-1(+)* is required in both fusing partners in *C. elegans*. The strongest evidence for this conclusion comes from the instances in which adjacent eff-1(−) cells do not fuse with only one eff-1(+) cell (Figure 7D; red lines).

Taken together, our results in developing *C. elegans* mosaic embryos and in transfected insect cells are consistent with the homotypic model.

Discussion

We have shown that the *C. elegans* EFF-1 transmembrane proteins, expressed at the surface of insect cells, initiate cell fusion and produce multinucleate syncytia. We found that reconstituted EFF-1 drives fusion via the same key intermediates as those in viral and intracellular fusion. EFF-1 forms complexes at the surface of the membranes, analogously with many viral and intracellular fusogens. However, the basic design of the EFF-1 fusion machinery is essentially different from that of established fusogens. While viral fusogens are located at one of the fusing membranes (Daniels et al., 1985; Doms et al., 1985; Gething et al., 1986; Kuhn et al., 2002; Blumenthal et al., 2003; Tamm et al., 2003; Earp et al., 2005; Kielian and Rey, 2006), EFF-1 is required in both fusion partners. EFF-1 is also distinct from SNARE-dependent intracellular fusion, in which two fusing membranes carry different but complementary sets of protein fusogens (Weber et al., 1998; Hu et al., 2003; Jahn et al., 2003; Bonifacino and Glick, 2004). Thus, we provide evidence supporting the finding that EFF-1-mediated fusion is, in general, homotypic both in a cell culture system and in tissues within *C. elegans* embryos. Homotypic relationships might reflect homophilic interactions between complexes of EFF-1 proteins expressed at two fusing membranes. Alternatively, it is still conceivable that EFF-1-mediated homotypic fusion involves additional proteins recruited by EFF-1 and conserved in evolution, at least from nematodes to insects.

Homotypic machinery may provide better control of a developmentally regulated fusion event than what is required for heterotypic virus-host cell fusion during infection. For example, homotypic fusion may prevent fusion with cells at the edges of a multinucleate cell, allowing better control of syncytium size and shape. This mechanistic aspect of cell fusion during syncytium formation is critical for the normal development of many organs in nematodes and in the formation of diverse tissues in mammalian organs as diverse as muscles, bones, placenta, and eye (Kuszak et al., 1985; Cross et al., 1994; Vignery, 2000; Abmayr et al., 2003; Podbilewicz, 2006). Time-lapse microscopy shows that the time required to complete syncytium formation in culture is comparable to the time required for a cell-cell fusion event in *C. elegans* embryos (T. Gattegno, B.P., L.V.C., et al., unpublished data). There appears to be a competition between cytokinesis and syncytiogenesis in EFF-1-expressing Sf9 cells. Analogously to stem cell fusion followed by transdifferentiation in mammals (Terada et al., 2002; Ying et al., 2002; Alvarez-Dolado et al., 2003; Shi et al., 2004), multinucleate insect cells expressing EFF-1 on their surface have been observed to divide and migrate in culture (Movie S2). Fused Sf9 cells appear to die days after fusion, like many syncytial tissue culture cells and unlike syncytia in *C. elegans* and other animals that stop dividing and undergo terminal differentiation and normal aging. In contrast, viruses, oncogenes, and mutated tumor suppressor genes can contribute to carcinogenesis by fusing cells (Duelli et al., 2005). Future studies will determine whether the homotypic organization of EFF-1 fusion machinery is shared by other developmental cell fusion reactions. We propose that this cell-cell fusion process gives better control of the size and shape of the syncytia by preventing fusion with neighboring cells. We anticipate that EFF-1 expression in other heterologous systems may be used to fuse cells, with potential applications for gene therapy and manipulation of stem cell fates.

Experimental Procedures

Transformation of Sf9 Cells

Sf9 cells were grown to ~50% confluency on 35 × 10 mm tissue culture plates as recommended by manufacturers. Cells were transfected with cellfectin and with plasmid at 1 μg/ml (either pIZT-Empty vector, pIZT-eff-1A, pIZT-eff-1B, pIZT-eff-1C, or pIZT-eff-1EC), as recommended by Invitrogen, and were analyzed at different times from 18 to 96 hr posttransfection.

Cell Fusion Assays

To assay syncytium formation and to correlate it with the expression of GFP reporter present in all plasmids used for the transfection, we

labeled cell nuclei with Hoechst (1 μg/ml, H3570, Molecular Probes) for 10 min at 22°C. We obtained GFP(+) fluorescence (transfected cells) and phase-contrast images for at least ten randomly selected fields of view by using fluorescence microscopy (Olympus IX70) and a cooled CCD camera Photometrics CoolSNAP-fx (Roper Scientific). Using ImageJ software, we determined the ratio of the number of nuclei in multinucleate cells to the total number of nuclei in the field. We also determined this ratio for GFP(+) cells. To minimize the effects of variability in the levels of EFF-1 expression and fusion from day to day, in the same figure we show means ± standard errors of at least seven experimental replicates from the same transfection. Each experiment presented was repeated at least three times, and all functional dependencies reported were observed in each experiment.

For the content mixing fusion assay, the cells were lifted and labeled either with Orange Cell Tracker (Cat. # C34551, Molecular Probes) or with Blue CMAC Cell Tracker (Cat. # C2110, Molecular Probes) 18 hr posttransfection. Fusion was detected as the appearance of multinucleate cells containing both probes after overnight incubation. Note that fusion events between the cells labeled with the same probe were not counted in this approach; the fusion efficiency detected with this approach was underestimated. The highest fusion extent detected with two-color assay was ~17%, versus extents of ~50% detected by scoring of syncytia.

To compare the redistribution of membrane and content probes in the hemifusion experiments, we labeled some lifted cells with Blue CMAC Cell Tracker and some cells with the membrane probe DiI by using cell-labeling solution from the Vybrant Multicolor cell-labeling kit (Cat. # V22889, Molecular Probes) as described by the manufacturer. In hemifusion experiments, we monitored fusion as early as 1 hr after coplating membrane-labeled and content-labeled cells. Cells labeled with different content probes were coplated and incubated together for 1–2 hr.

Statistical Tests

We evaluated the significance of differences between mean values by using the t test function of SigmaPlot 2000, and we evaluated the probability that EFF-1 present in either one or two bound membranes fuses membranes with the same efficiency by binomial distribution analysis with the Excel BINOMDIST function (cumulative form).

Fusion between Transfected and Innocent Sf9 Cells

Four hours after the application of DNA and Cellfectin, the transfected cells were washed, and innocent Sf9 cells labeled with Orange Cell Tracker were added. After a 36 hr coincubation, we analyzed the cells with fluorescence microscopy. Fusion between transfected (green GFP fluorescence) and innocent (orange) Sf9 cells would be detected as the appearance of syncytia labeled by both probes.

Nematode Strains

Bristol N2 was used as the wild-type (Brenner, 1974). The following strains were used:

eff-1(ok1021)/mIn1(mIs14 dpy-10[e128]) II
The *eff-1(ok1021)* deletion strain obtained from the *C. elegans* consortium and outcrossed six times
eff-1(ok1021)II;jcIs1[ajm-1::gfp, pRF4] IV
eff-1(ok1021) II;jcIs1 IV; hyEx99(pTG96[sur-5:gfp],pJE8[*eff-1* genomic rescuing fragment]).

Mosaic Analysis in *C. elegans*

Mosaic analysis was carried out as previously described (Yochem et al., 1998; Yochem and Herman, 2003). The extrachromosomal array *hyEx99* was obtained by microinjection of the *eff-1* genomic rescuing fragment (pJE8, 3 ng/ml) along with the *sur-5::gfp* nuclear marker (pTG96, 100 ng/ml) to N2 worms. One line carrying the transgene was crossed with *eff-1(ok1021); jcIs1* nematodes. Extrachromosomal transmittance was <50%.

Adult, gravid hermaphrodites were treated with hypochlorite, and the eggs were floated on sucrose. Embryos and L1 larvae were mounted on agar pads for analysis. For observation of larvae, agar pads contained 10 mM Na azide. Mosaic worms were scored on the basis of partial loss of GFP-containing nuclei of defined cells (Sulston et al., 1983). The following cells were scored for the mosaic phenotype: 2 hyp6 cells, hyp5R, H0R, H1R as ABarpa descendants; H2L, V1L, V2L, V4L, V6L, H2R, V1R, V2R, V4R, V6R as ABarpp descendants; hyp2V as ABalp descendant; 2 hyp6 cells, hyp5L, H0L, H1L, hyp4 as ABplaa descendants; P1/2L, P3/4L, P5/6L, P7/8L, P9/10L, P11/12L, V3L as ABplap descendants; excretory cell, hyp10, repVL as ABplp descendants; hyp4, hyp6, P1/2R, P3/4R, P5/6R, P7/8R, P9/10R, P11/12R, V3R as ABpra descendants; hyp10, repVR as ABprp descendants; body muscles as MS descendants; intestinal cells as E descendants; and hyp7 cells as C descendants. In the embryo, there are 11 hyp7 cells that originate from AB and 12 that originate from C, all of which were used to screen for *eff-1* mosaics (Sulston et al., 1983; Podbilewicz and White, 1994).

We screened over 500 transgenic nematodes. Approximately 200 worms showed a full rescued phenotype, including full epidermal fusion events. Six mosaics were obtained; two of them had a complete loss of AB and one additional loss, in C and in EMS, respectively. One worm had a loss in Aba, and three had multiple losses of the array in AB.

Epidermal cells' nuclei were detected with respect to the apical junction marker (AJM-1::GFP), and their precise locations were determined (Sulston and Horvitz, 1977). All hypodermal cells of each mosaic worm were analyzed and screened for the disappearance/presence of nuclei and for a corresponding junction or a fusion event between each pair (Figure S2).

Examples in which adjacent eff-1(−) cells do not fuse with only one eff-1(+) cell (Figure 7D; red lines) provide good evidence for the conclusion that *eff-1(+)* is required in both fusing partners in *C. elegans*. This is because if some cells express a green nuclear marker prior to fusion while others do not, then it is likely that all nuclei in the syncytium will display the nuclear marker, as the marker is synthesized in the cytoplasm and transported to all nuclei.

Supplemental Data

Supplemental Data showing Experimental Procedures, a summary of *eff-1* mosaics, two figures, and two movies are available at http://www.developmentalcell.com/cgi/content/full/11/4/471/DC1/.

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